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Activation of cAMP-Responsive Genes by Stimuli That Produce Long-Term Facilitation in Aplysia Sensory Neurons

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Summary

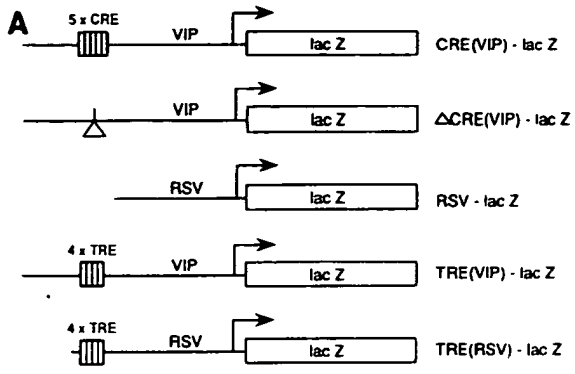
One of the hallmarks of long-term memory in both vertebrates and invertebrates is the requirement for new protein synthesis. In sensitization of the gill-withdrawal reflex in Aplysia, this requirement can be studied on the cellular level. Here, long-term but not short-term facilitation of the monosynaptic connections between the sensory and motor neurons requires new protein synthesis and is reflected in an altered level of expression of specific proteins regulated through the cAMP second-messenger pathway. Using gene transfer into individual sensory neurons of Aplysia, we find that serotonin (5-HT) induces transcriptional activation of a *lacZ* reporter gene driven by the cAMP response element (CRE) and that this induction requires CRE-binding proteins (CREBs). The induction by 5-HT does not occur following a single pulse, but becomes progressively more effective following two or more pulses. Moreover, expression of GAL4–CREB fusion genes shows that 5-HT induction requires phosphorylation of CREB on Ser^{119} by protein kinase A. These data provide direct evidence for CREB-modulated transcriptional activation with long-term facilitation.

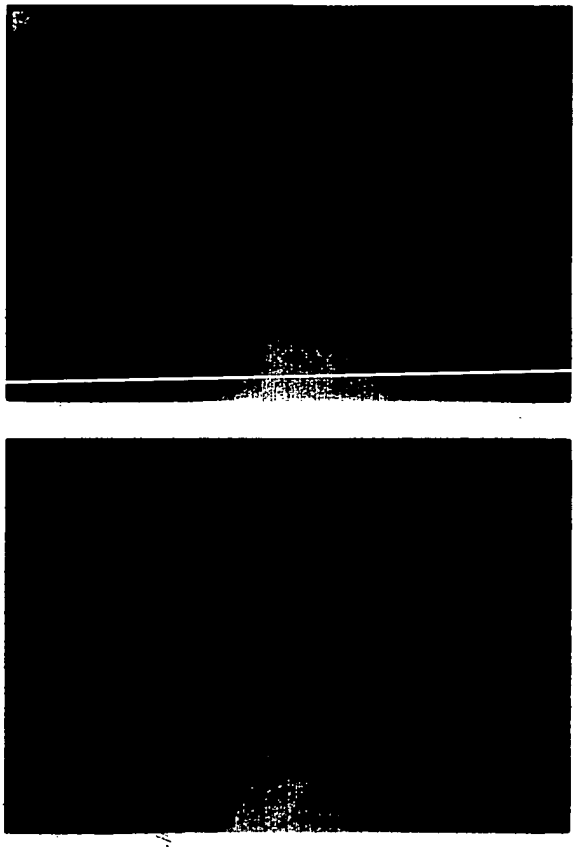
Introduction

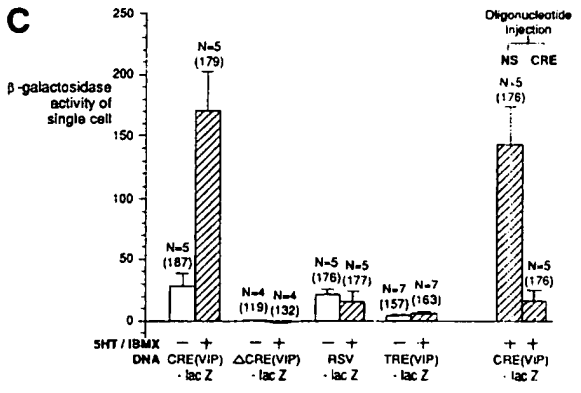
Studies of learning in experimental animals indicate that, even though the transition from short-term memory (which lasts seconds to minutes) to long-term memory (which lasts days, weeks, or longer) is frequently a graded function of the number of training trials, long-term memory can nevertheless be dissociated from short-term memory by inhibiting protein and mRNA synthesis during training (Barondes, 1975; Davis and Squire, 1984). In the gill-withdrawal reflex of Aplysia, the transition from short- to long-term memory can be examined on both the behavioral and cellular level (Montarolo et al., 1986; Schacher et al., 1988; Bailey and Chen, 1983; Frost et al., 1985). The monosynaptic connections, between the siphon sensory neurons and gill motor neurons, can be studied both in the intact animal and in cell culture. These connections undergo an increase in synaptic effectiveness, of which the amplitude and duration are a function of the number of behavioral reinforcing stimuli to the tail in the intact animal; in cell culture it is a function of the number of applications of 5-hydroxytryptamine (5-HT), a modulatory transmitter released by tail stimuli (Montarolo et al., 1986; Glanzman et al., 1990; Rayport and Schacher, 1986). A single tail stimulus or a single (5 min) pulse of 5-HT produces short-term facilitation lasting minutes, whereas five pulses (over a 1.5 hr period) or continuous application of 5-HT for 1.5 or 2 hr elicits long-term facilitation lasting 1 or more days. In both the behavioral reflex and in the facilitation of the monosynaptic component, there is a parallel requirement for protein and mRNA synthesis for long-term but not for short-term facilitation (Montarolo et al., 1986; Castellucci et al., 1989).

In earlier experiments, Barzilai et al. (1989) found that five repeated pulses of 5-HT or of cAMP, a second messenger activated by 5-HT, induced changes in the synthesis of proteins in the sensory neurons, as detected on quantitative two-dimensional gels. The induction of these proteins may reflect the regulated transcription of cAMP-inducible genes since the 5-HT-induced synthesis of these proteins was blocked by the general transcription inhibitor actinomycin D. Moreover, Dash et al. (1990) were able to block long-term facilitation without affecting short-term facilitation by injecting into sensory neurons an oligonucleotide encoding the cAMP response element (CRE), TGACGTCA. These results suggested that long-term facilitation may require the activation, by 5-HT, of cAMP-inducible genes and that the CRE oligonucleotide prevented CRE-binding protein (CREB)-related transcriptional factors from binding to these genes.

To test this idea directly and to explore the steps whereby cAMP regulates genes important for long-term facilitation, we have developed a highly efficient gene transfer system, based on DNA microinjection, designed to examine the inducible expression of specific genes in mature, differentiated sensory neurons of the intact Aplysia nervous system (Kaang et al., 1992). This methodology has allowed us to address two questions: can the facilitating transmitter, serotonin, activate transcription in the sensory neurons, and, if so, does serotonin activate transcription through the protein kinase A (PKA)-mediated phosphorylation of CREB-related transcription factors that bind to the CRE? We now report, based on gene transfer into individual Aplysia sensory neurons, that repeated pulses of 5-HT result in the induction of a reporter plasmid driven by the CRE. The induction is graded. It does not occur following a single pulse of 5-HT. In addition, 5-HT does not induce a TPA response element (TRE)-driven reporter gene. Moreover, coexpression of a reporter (GAL4–CAT) and a wild-type or mutant GAL4–CREB fusion protein reveals that the transcription induced by 5-HT requires that CREB be phosphorylated







on Ser[119] by PKA. Thus, in the sensory neuron the protein synthesis–dependent changes in long-term facilitation can now be traced directly from the serotonin receptors coupled to adenylyl cyclase at the membrane to CRE-driven genes in the nucleus.

Results

Serotonin Drives CRE-Mediated Transcription in Neurons

Stimulation of Aplysia sensory neurons with serotonin results in elevation of intracellular cAMP and the consequent activation of PKA (Kandel and Schwartz, 1982). In mammalian cells, sustained elevation of cAMP results in a rapid and transient transcriptional induction of genes that share the CRE (Roesler et al., 1988; Montminy et al., 1990). Because the CRE will also confer cAMP inducibility when placed upstream of reporter genes, these reporter constructs can serve as an assay system for transcriptional induction by cAMP (Montminy et al., 1986; Tsukada et al., 1987). We therefore injected into Aplysia sensory neurons the DNA construct CRE(VIP)-*lacZ*, which contains five copies of the CRE upstream of the vasoactive intestinal peptide (VIP) promoter driving the *lacZ* reporter gene (Figure 1A). We then measured, both qualitatively (Figure 1B) and quantitatively (Figure 1C; Table 1), the levels of *lacZ* expression driven by the CRE in response to stimulation by cAMP or 5-HT.

We first exposed the sensory neurons to a cAMP analog (8-[4-chlorophenylthio]-cAMP, CPT-cAMP) and found that it produced a 3.4-fold induction of *lacZ* expression (Table 1). We then exposed the neurons to five repeated (5 min) pulses of 5-HT over a period of 1.5 hr, a protocol that produces long-term synaptic facilitation, and found a 3.9-fold induction of *lacZ* ex-

Figure 1. Induction of CRE(VIP)-*lacZ* Expression by 5-HT-IBMX

(A) DNA constructs. CRE(VIP)-*lacZ* contains 2146 bp of VIP upstream region (−2000 to +146) with five copies of VIP CRE (−95 to −70) fused to the E. coli *lacZ* gene. ΔCRE(VIP)-*lacZ* has a deletion of the CRE from the VIP upstream region. RSV-*lacZ* was used as a constitutive expression reporter. TRE(RSV)-*lacZ* and TRE(VIP)-*lacZ* contain four copies of AP-1-binding sequence (GTGACTCAGCGCG) upstream of the RSV and VIP promoters, respectively.

(B) Serotonin drives CRE(VIP)-*lacZ* expression in Aplysia sensory neurons. Forty cells per sensory cluster in the pleural ganglion were injected with the CRE(VIP)-*lacZ* DNA construct and stained with X-gal. Upper panel is control and lower panel is treated with 5-HT-IBMX.

(C) Expression of E. coli *lacZ* in Aplysia sensory neurons under the control of CRE or TRE enhancer sequences. 5-HT-IBMX stimulates expression of CRE(VIP)-*lacZ*, in contrast with ΔCRE(VIP)-*lacZ*, RSV-*lacZ*, and TRE(VIP)-*lacZ*. The 5-HT-IBMX-induced expression of CRE(VIP)-*lacZ* is inhibited by coinjected oligonucleotides encoding CRE but not nonspecific sequence (NS). P value calculated from two-tailed paired t test. (N is the number of injected sensory clusters. The value in parentheses is the total number of injected sensory neurons.) Histograms show the mean value and standard error of the mean of β-galactosidase activity (fluorescence unit) per sensory neuron.

Table 1. Induction of CRE(VIP)-*lacZ* and TRE-*lacZ* Expression

DNA Construct	Drug	Treatment[a]	Induction Fold[b]	P Value in t Test[c]	Number of Tested Animals (Injected Sensory Neurons)
CRE(VIP)-*lacZ*	CPT-cAMP (1 mM)	Continuous (5 hr)	3.4	0.004	4 (263)
	IBMX (500 μM)	Continuous	2.4	0.072	5 (398)
	5-HT (100 μM)	Continuous	5.6	0.046	5 (309)
	5-HT-IBMX	Continuous	6.0	0.006	5 (366)
	5-HT	Five pulses	3.9	0.017	6 (417)
	5-HT-IBMX	Five pulses	4.6	0.014	9 (620)
TRE(VIP)-*lacZ*	5-HT	Continuous	1.4	>0.05	7 (320)
	TPA or PDBu (4 μM)	Continuous (5 or 8 hr)	1.1	>0.05	5 (232)
TRE(RSV)-*lacZ*[d]	5-HT	Continuous (8 hr)	0.8	>0.05	6 (120)
	TPA (1 μM)	Continuous (15 hr)	0.6	0.08	6 (120)
	PDBu (4 μM)	Continuous (8 hr)	0.8	>0.05	5 (100)

PDBu, phorbol 12,13-dibutyrate.
[a] Continuous treatment indicated in parentheses means the treatment without washing before harvesting.
[b] Induction fold was calculated by dividing the average of β-galactosidase activity of treated single sensory neurons by that of control.
[c] Two-sided t test in paired samples.
[d] MUG assay was done at room temperature for 30 min.

pression (Table 1). Similarly, continuous stimulation throughout the 2 hr period with 5-HT, another protocol that also produced long-term facilitation (S. Schacher and M. Ghirardi, personal communication), also induced *lacZ* expression by 5.6-fold with 5-HT alone and by 6-fold with 5-HT and isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor (Figure 1C, Table 1). Thus, repeated pulses or prolonged exposure to 5-HT stimulates gene expression and this stimulation can be simulated by elevating levels of cAMP.

This increase in *lacZ* expression stimulated by 5-HT is a transcriptional induction mediated through the CRE sequence. Deletion of the CRE from CRE(VIP)-*lacZ*, ΔCRE(VIP)-*lacZ*, abolishes both basal and inducible expression (Figures 1A and 1C). Moreover, a constitutively expressed construct, Rous sarcoma virus promoter (RSV)-*lacZ*, lacking the CRE, also showed no increase in *lacZ* expression after stimulation with 5-HT and IBMX.

The CRE (TGACGTCA) is closely related to the TRE (TGACTCA) that was originally defined as the binding site for activator protein 1 (AP-1). In mammalian cells, many transcription factors that contain the basic region-leucine zipper motif bind both CRE and AP-1 but with different affinity; members of the AP-1 family preferentially bind the AP-1 site and members of the CREB/ATF family bind the CRE (Nakabeppu et al., 1988; Fink et al., 1991; Sassone-Corsi et al., 1990; Hai and Curran, 1991). To explore the cross talk between the pathways that activates the CRE and the TRE, we tested whether the TRE could drive expression of *lacZ* in sensory neurons and if this expression could be stimulated by 5-HT or phorbol esters (Table 1; Figure 1). The TRE site enhanced the basal level of expression of the VIP promoter (TRE(VIP)-*lacZ*) and RSV promoter (TRE(RSV)-*lacZ*) (Angel et al., 1987; Lee et al., 1987); however, neither 5-HT nor phorbol esters (TPA or phorbol 12,13-dibutyrate) could further stimulate this expression. The failure of phorbol ester to up-regulate gene expression is unlikely to result from the short period of drug treatment, since longer incubation times with phorbol ester (up to 18 hr) also failed to stimulate reporter genes (Table 1). Moreover, since 5-HT or phorbol ester can activate Aplysia protein kinase C (Sossin and Schwartz, 1992), there might be some cell type-specific restriction to phorbol-mediated TRE activation in sensory neurons, similar to that reported in mammalian cells (Chiu et al., 1989). These results show that the factors responsible for CRE induction with 5-HT can discriminate between the TRE sequence and the CRE sequence. One interpretation of these results is that factors that preferentially bind the CRE may be more important for the 5-HT stimulation of transcription in sensory neurons than those that bind the AP-1 site.

CRE-Mediated Transcription Requires a Transactivator(s) That Binds to CRE

Does the stimulation of *lacZ* transcription mediated by 5-HT depend on the binding of transcription factors to the CRE sequence? To address this question, we carried out competition experiments similar to those of Dash et al. (1990), who found that injection of the CRE in the sensory neurons selectively titrated out CREB-related proteins and blocked the long-term facilitation without affecting short-term facilitation. We therefore microinjected CRE(VIP)-*lacZ* with either an oligonucleotide that encodes the wild-type CRE sequence and binds CREB-like factors or with a mutant oligonucleotide that does not bind these factors. The wild-type oligonucleotide blocked the serotonin-induced expression of *lacZ*, whereas the mutant oligonucleotide produced no inhibition (Figure 1C). These experiments suggest that the induction mediated by

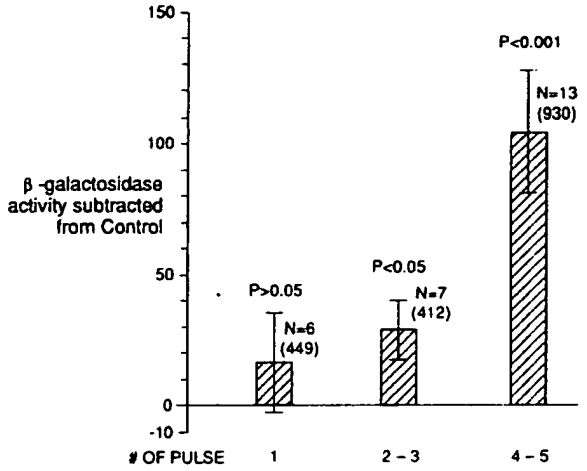


Figure 2. Induction of CRE(VIP)-*lacZ* Expression Is a Graded Response to 5-HT-IBMX

The sensory clusters injected with CRE(VIP)-*lacZ* were treated with 5 min pulses (one, two or three, and four or five) of 5-HT-IBMX, and histograms display the mean value and standard error of the mean of the difference in *lacZ* activity between stimulated and control sensory neurons.

5-HT requires positively acting cellular factors that interact with the CRE and provides direct evidence that serotonin can stimulate transcription of genes containing the CRE sequences.

Transcriptional Activation Correlates with Long-Term Facilitation

Is the activation of transcription by 5-HT graded in a linear way, or does it show cooperativity, suggesting a discrete threshold? In assessing this question we were influenced by the finding that a single (5 min) pulse of serotonin only elicits short-term facilitation (Montarolo et al., 1986; Castellucci et al., 1989). To generate significant long-term facilitation, four or five pulses are required. Using the same pulse protocol, we found that a single pulse of 5-HT does not stimulate expression from CRE(VIP)-*lacZ* ($p > 0.05$) (Figure 2), whereas four to five pulses of 5-HT produced a 6.7-fold stimulation ($p < 0.001$). An intermediate number of pulses (two or three) gave a much smaller (2.2-fold, $p < 0.05$) amount of stimulation, suggesting that there is a nonlinear relationship between the number of 5-HT pulses and the level of transcriptional induction and facilitation. Thus, by simply varying the number of pulses, it is possible for a modulatory transmitter such as 5-HT to select between a transient program of neural action restricted to the cytoplasm, namely, short-term facilitation, or to extend the duration of its action through the recruitment of a genomic program in long-term facilitation.

Serotonin Activates GAL4-CREB Transcription Factors by Phosphorylation of Ser^{119}

Studies in mammalian cells show the CRE interacts with a superfamily of transcription factors (Hai et al., 1989; Hai and Curran, 1991) characterized by dimerization through the leucine zipper motif (as occurs in the CREB/ATF and Fos/Jun families of proteins). CREB is the best-characterized cAMP-regulated factor (Hoeffler et al., 1988; Gonzalez et al., 1989; Gonzalez and Montminy, 1989; Yamamoto et al., 1988; Lee et al., 1990; Berkowitz and Gilman, 1990; Meinkoth et al., 1991; Dwarki et al., 1990). In Aplysia, gel mobility shift and DNAase I footprinting assays show that nervous system extracts bind the CRE and that extracts enriched by CRE affinity chromatography contain a 43 kd protein that cross-reacts with an antiserum to mammalian CREB. It is therefore likely that Aplysia expresses a CREB-like protein (Dash et al., 1990). Thus, we next focused on CREB as a potential target for regulation by 5-HT and asked: does 5-HT regulate transcription in sensory neurons via phosphorylation of CREB? For these experiments we have microinjected cloned mammalian CREB genes with mutations that prevent CREB phosphorylation and assayed their ability to regulate transcription of a second reporter gene in response to 5-HT.

We took advantage of the modular construction of the CREB transcription factors/ATFs, which comprise two major functional domains, the DNA-binding domain and the activation domain (Dwarki et al., 1990; Gonzalez and Montminy, 1989; Lee et al., 1990). We used a plasmid that encoded a hybrid protein consisting of the yeast GAL4 DNA-binding domain and the mammalian CREB activation domain (Figure 3A). This plasmid was coinjected with a reporter construct (GAL4–CAT) that contains DNA-binding sites for GAL4 that drive the expression of a CAT reporter gene (Figure 3A). Thus, transcription of CAT is induced when the activation domain of GAL4–CREB is appropriately phosphorylated, and this protein specifically binds the GAL4 upstream elements in the GAL4–CAT plasmid. Since the yeast GAL4-binding site is not likely to be recognized by Aplysia transcription factors, this approach reduces the complexity of the transcription assay and thereby allows the analysis of a specific CREB molecule in the context of the Aplysia sensory neuron.

We first tested whether exposure of sensory neurons to 5-HT induced expression of CAT in neurons that had been coinjected with both the GAL4–CREB and the GAL4–CAT constructs. We found that 5-HT induced a robust, 7.5-fold stimulation of expression (Figures 3B and 3C), indicating that the hybrid yeast/mammalian transcription factor is capable of being activated by the signal transduction pathway initiated in Aplysia sensory neurons by stimulation with 5-HT.

Within the transactivation domain of CREB is a single phosphorylation site (Ser^{119}) essential for transcriptional activation mediated by PKA (Lee et al., 1990) or Ca^{2+}/calmodulin-dependent kinase (CaM kinase) (Dash et al., 1991; Sheng et al., 1991). This phosphorylation site (Ser^{119}) in the human CREB-327 (Hoeffler et al., 1988) is equivalent to that (Ser^{133}) in the rat CREB-341 (Gonzalez et al., 1989). cAMP-dependent phosphory-

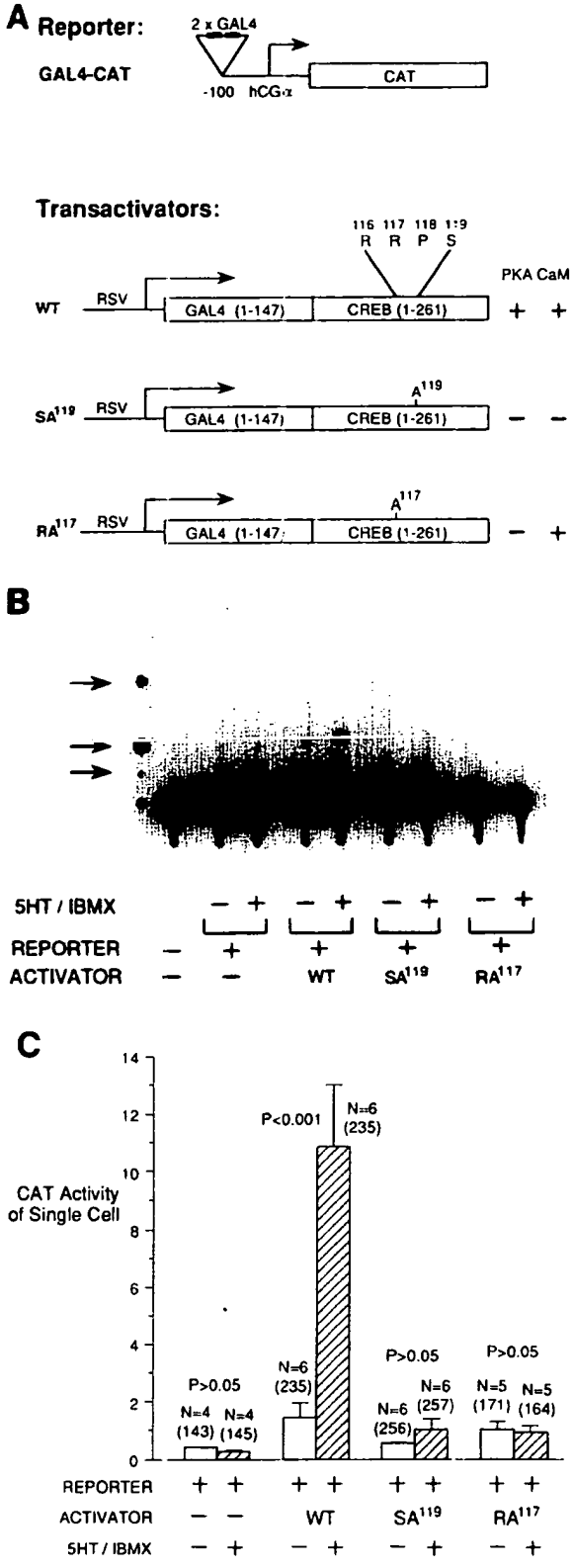


Figure 3. 5-HT-IBMX Leads to Activation of CREB through the Phosphorylation of PKA

(A) DNA constructs used for transactivation experiments. DNA-binding domain of yeast GAL4 transcription factor (amino acids 1–147) fused to wild-type or mutated forms of mammalian CREB transactivation domains (amino acids 1–261). Wild-type and mutated phosphorylation consensus sequences are indicated in single-letter amino acid code above the constructs. The wild-type sequence (RRPS) is a phosphorylation substrate for both protein kinase A (PKA) and CaM kinase at Ser[119]. SA[119] contains a substitution of Ser[119] with Ala[119], preventing phosphorylation by either kinase. RA[117] contains a substitution of Arg[117] with Ala[117], which abolishes the PKA site but leaves the CaM kinase consensus phosphorylation sequence intact. GAL4–CREB fusion proteins were constitutively expressed in pNEXδ vector and injected with the reporter construct (GAL4–CAT) containing two copies of the GAL4 site upstream of the human chorionic gonadotropin α subunit gene driving the CAT gene.

(B) Fluorescent CAT assay of CREB-regulated expression. The wild-type (WT) GAL4–CREB transactivator enhances 5-HT–IBMX-mediated expression of CAT, whereas mutant (SA[119] and RA[117]) GAL4–CREB shows no enhancement. Pairs of sensory clusters were injected and treated in parallel; each lane represents CAT activity of one sensory cluster. The arrows indicate acetylated products. The first column shows positive control (Molecular Probes, Inc.). The negative image is from thin layer chromatography of a fluorescent CAT assay.

(C) Quantitative analysis of 5-HT-regulated CREB transactivation. CAT activity is expressed as the percentage of substrate acetylated (× 10^3). Histograms show pooled data: N is the number of animals, and the value in parentheses is the number of injected neurons. Mean, standard error of the mean, and P value from two-tailed paired t test are shown.

lation of these sites has been demonstrated to be important for the activity of CREB-327 (Lee et al., 1990) and CREB-341 (Gonzalez and Montminy, 1989). Therefore, in addition to testing the ability of 5-HT to activate transcription via CREB, mutations introduced into GAL4–CREB allowed us to examine the requirement for phosphorylation on Ser[119] by PKA and CaM kinase.

To test for this requirement, we introduced a mutation into GAL4–CREB that converted Ser[119] to Ala[119] (SA[119]) (Figure 3A), thereby abolishing the phosphorylation site of PKA and CaM kinase. In contrast with the activation of transcription we had obtained with the wild-type CREB activation domain, we now observed that mutations of Ser[119] to Ala[119] abolished stimulation with 5-HT. This suggests that the transcription mediated by 5-HT requires that CREB be phosphorylated on Ser[119].

Since phosphorylation on Ser[119] is necessary not only for transcription mediated by PKA but also for Ca^{2+}-dependent regulation by CaM kinase (Dash et al., 1991; Sheng et al., 1991), we next attempted to dissect the contribution of these two kinase pathways to the activation of CREB. The CREB phosphorylation site is Arg-Arg-Pro-Ser[119], whereas the consensus phosphorylation site for PKA is Arg-Arg-Xaa-Ser (Xaa indicates any amino acid) and that for CaM kinase is Arg-Xaa-Xaa-Ser(Thr) (Pearson et al., 1985; Kemp et al., 1977; Colbran et al., 1989). We therefore introduced a mutation that leaves Ser[119] intact but inactivates the PKA phosphorylation site but not the CaM kinase phosphorylation site. By constructing the CREB mutant Arg-Ala[117]-Pro-Ser[119] (RA[117]), we could remove the PKA recognition site, yet leave the CaM kinase motif intact. When RA[117] was coinjected with the reporter,

we found no significant stimulation of transcription by 5-HT, indicating that only PKA is likely to phosphorylate and activate CREB. Although this result suggests that a CaM kinase pathway does not contribute to CREB activation by 5-HT, we cannot, based on these experiments alone, eliminate the alternative possibility that this failed induction may result from CREB mutant RA[117] being a poorer substrate for CaM kinase than wild-type CREB.

Discussion

The data we present here provide direct evidence that long-term facilitation induced by 5-HT involves activation of CRE-inducible genes and that the induction of these genes by the cAMP cascade is initiated through phosphorylation of CREB at Ser[119]. In earlier physiological experiments, Dash et al. (1990) found that long-term facilitation was blocked by injection of the CRE oligonucleotides designed to titrate CREB. Our data, taken together with those of Dash et al., indicate that the phosphorylation of CREB-related proteins and the consequent activation of transcription represent a key component of the switch for extending the short-term process for presynaptic facilitation, which is independent of protein synthesis, into the long-term process, which requires gene expression. Whereas the results of Dash et al. (1990) could not distinguish between constitutive or induced activation of cAMP-inducible genes, our data clearly indicate that 5-HT leads to induction of CRE-driven genes.

We find that the graded nature of long-term facilitation may, in part, be attributable to the graded regulation of transcription by CREB. An attractive feature of a graded mechanism is that it provides more flexibility than an on/off switch characteristic of cellular differentiation. Further analysis of the graded response will require an exploration of the thresholds and the respective time courses of the several steps involved in the transcriptional induction: the increase in cAMP, the activation of PKA, the phosphorylation of CREB, and gene induction. As a first step in this direction, we have attempted to examine the increase in cAMP and activation of PKA produced by single and repeated (or prolonged) exposure to 5-HT (Bacskai et al., 1993). These studies suggest that a single exposure to 5-HT primarily increases the level of cAMP and activates PKA in the process of the sensory neurons. To activate PKA in the cell body so as to lead to the translocation of the catalytic subunit to the nucleus, repeated or prolonged exposure to 5-HT or to cAMP is required (Bacskai et al., 1993). Although these several experiments provide direct evidence that stimuli that produce long-term facilitation can activate transcription by means of PKA, they do not exclude the possibility that other second-messenger pathways for gene activation may also be important.

In a larger sense these studies provide direct evidence that a conventional chemical transmitter, acting through a second-messenger pathway, can produce synaptic actions of widely different time courses as a function of the number of times it is applied. A single pulse of 5-HT activates the cAMP-dependent protein kinase, so that it phosphorylates substrates in the cytoplasm, leading to an enhancement of transmitter release for a period of minutes. Four or five repeated pulses of 5-HT or exposure to drugs that increase cAMP are capable of translocating the catalytic subunit to the nucleus (Bacskai et al., 1993), where it can phosphorylate CREB (and presumably other transcriptional activator proteins), which in turn can activate CRE-driven genes to prolong the enhancement of transmitter release for 1 or more days. Thus, the distinction between short- and long-term facilitation derives in part from the ability of 5-HT to select, based on the number of stimuli presented, between a cellular program involving only the cytoplasmic actions of the cAMP pathway and a program that involves, in addition, the recruitment of nuclear transcription factors. Moreover, by activating gene expression and becoming competent to stimulate synaptic growth, transmitters such as 5-HT take on the properties of growth factors. It will now be important to identify endogenous Aplysia genes regulated by 5-HT and to study their physiological regulation by CREB and other transcription factors. The ability to express cloned genes in the mature nervous system of Aplysia, using either the constitutive (Kaang et al., 1992) or inducible expression vectors applied in this study, should allow further analyses of the physiological roles of genes induced during long-term facilitation.

Gene induction with long-term synaptic facilitation seems to be a general property of several types of learning in both the vertebrate and the invertebrate nervous systems. In the mammalian hippocampus a robust form of synaptic enhancement known as long-term potentiation, which shares several physiological properties with long-term facilitation in Aplysia, is thought to be a contributing component to the neural mechanisms for explicit forms of learning. Induction of long-term potentiation is associated with increased expression of mRNA encoding both transcription factors (Cole et al., 1989; Wisden et al., 1990) as well as effector proteins potentially important for growth (Qian et al., 1993). These findings suggest that in mammals as well as in Aplysia long-term changes in synaptic plasticity may also be linked to gene induction, and that one of the functions of this induction is to initiate synaptic growth. Using gene transfer technology, either by stably integrating genes in the germline or by transient expression, as used in this study, it should be possible to analyze how physiological stimulation regulates expression of these genes. Moreover, this approach can be used to determine the causal role played by induced genes and specifically to determine their contribution to synaptic growth.

Experimental Procedures

Construction of Expression Vector

The expression plasmid pNEX (Kaang et al., 1992) was modified to pNEXδ by deletion of a HindIII site 5′ of the promoter/enhancer region, facilitating the subcloning of GAL4–CREB or *lacZ* at the remaining HindIII site in the polylinker.

CRE- or TRE-Containing DNA Constructs

CRE(VIP)-*lacZ*, RSV-*lacZ*, and VIP-2.0Z were provided by Dr. J. S. Fink, and the upstream regions of promoters were verified by DNA sequencing. The single CRE sequence (−104/−70) was deleted from the VIP upstream region of VIP-2.0Z using the polymerase chain reaction (PCR) (Higuchi, 1989). In brief, amplification from the VIP-2.0Z template with a sense primer (5′-$^{-501}$TTATCTCTAG-AGCTTTT-$^{-485}$-3′) and an antisense primer containing sequences flanking and deleting the CRE (−69/−104) (3′-$^{-53}$CACAAAGTGCT-CTGAAA $^{-69/-104}$GCTTGAAGTTCTTACTT^{-170}-5′) generated a 413 bp fragment (denaturation at 94°C for 1 min, annealing at 44°C for 1 min, polymerization at 72°C for 1 min, 23 cycles). Next, a second fragment (1 kb) was derived by amplification from the VIP-2.0Z template with a sense primer (5′-$^{-117}$AAGAACTTCAAG$^{-105/-69}$TTT-CAGAGCACTTTGTGA^{-51}-3′) and an antisense primer (3′-GCGGT-TTTCTCCGGCGC-5′) in *lacZ* (denaturation at 94°C for 1 min, annealing at 50°C for 1 min, polymerization at 72°C for 1 min, 23 cycles). These two overlapping fragments were mixed, and recombinant PCR (denaturation at 94°C for 1 min, annealing at 42°C for 1 min, polymerization at 72°C for 2 min, 25 cycles) was performed to generate a 1.4 kb fragment; this fragment was then digested with NsiI and HindIII to release a 450 bp fragment used to replace the NsiI–HindIII CRE-containing sequence in VIP 2.0Z, resulting in ΔCRE(VIP)-*lacZ*. The sequence of the subcloned region was checked by the dideoxy method.

TRE(VIP)-*lacZ* was made by subcloning four copies of the AP-1 site (70 bp BSP106–PstI fragment) from 4×AP-1-RSV-Z into ΔCRE(VIP)-*lacZ* (AccI and NsiI site). TRE(RSV)-*lacZ* was made by subcloning *lacZ* gene into pNEXδ.

The competitor DNA fragments spanning −97 to −59 of the VIP upstream region were made by annealing sense and antisense synthetic oligonucleotides. Sense strand DNA sequences were GCATCCCATGGCCGTCATACTGTGACGTCTTTCAGAGCACC for wild-type CRE and GCATCCCATGGCAAGTGTACTGCTTAATCT-TTCAGACGACC for mutant CRE.

Transactivators and Reporter Constructs

The chimeric GAL4–CREB gene was taken from pZ1 (Lee et al., 1990) using HindIII and BclI and subcloned into HindIII- and BamHI-cleaved pNEXδ vector to make pNEXδ-WT.

SA^{119} Mutagenesis

Amplification from pNEXδ-WT with a sense primer (5′-CTTTCAA-GGAGGCCTGCCTACAGGAAAATTTTG-3′) and an antisense primer (5′-GACACCAGACCAACTGG-3′) generated a 500 bp fragment (denaturation at 94°C for 30 s, annealing at 51°C for 1 min, polymerization at 72°C for 40 s, 20 cycles); this fragment was digested with StuI and XbaI to produce a 400 bp fragment used to replace the StuI–XbaI sequence in pNEXδ-WT, resulting in pNEXδ-SA^{119} (SA^{119}).

RA^{117} Mutagenesis

Amplification from pNEXδ-WT complete with a sense primer (5′-ATCGAACAAGCATGCGA-3′) and an antisense primer (5′-CCT-GTAGGAAGGCGCCCTTGAAAGAATTTCCC-3′) containing a mutation in amino acid residue 117 from arginine to alanine (CCT→CGC) generated a 750 bp fragment (denaturation at 94°C for 1 min, annealing at 50°C for 1 min, polymerization at 72°C for 1 min, 25 cycles). Next, a second fragment (500 bp) was derived by amplification from the same template (500 bp) with a sense primer (5′-TCTTTCAAGGGCGCCTTCCTACAGGAAAATT-3′) containing the same mutation in amino acid residue 117, and an antisense primer (5′-GACACCAGACCAACTGG-3′) (denaturation at 94°C for 1 min, annealing at 50°C for 1 min, polymerization at 72°C for 1 min, 25 cycles). These two overlapping fragments were mixed, and recombinant PCR (denaturation at 94°C for 1 min, annealing at 50°C for 1 min, polymerization at 72°C for 1 min, 25 cycles) was performed to generate a 1.3 kb fragment; this fragment was then digested with XhoI and XbaI to release a 1.1 kb fragment used to replace the XhoI–XbaI sequence in pNEXδ-WT, resulting in pNEXδ-RA^{117} (RA^{117}). The sequences of all mutations and any misincorporation during PCR amplification were checked by the dideoxy DNA sequencing method. The reporter construct (GAL4–CAT) contains two copies of the GAL4 site upstream of the human chorionic gonadotropin α subunit gene driving the CAT gene (Lee et al., 1990), and the sequence of the upstream region was verified by DNA sequencing.

Microinjections and Preparation of Neuronal Extracts

Microinjections were performed as previously described (Kaang et al., 1992). DNA concentration in the microelectrode was 0.9 mg/ml for single plasmids, and for oligonucleotide competition experiments the oligonucleotide concentration was 2 mg/ml, resulting in 100-fold molar excess of oligonucleotide to plasmid CRE. Central nervous system from Aplysia californica (150–200 g, Marinus) was isolated as described by Byrne et al. (1978), and the desheathed sensory cluster in the pleural ganglion was exposed for microinjection by pinning the cerebropleural nerve and pedal ganglion. For quantitative analysis (Figure 1C), 35 neurons per cluster were injected within 5 min with the various DNA constructs (Figure 1A), except that 10 cells per cluster were injected with TRE(RSV)-*lacZ* DNA.

The DNA concentrations of coinjected transactivator and reporter were 0.08 μg/μl and 0.6 μg/μl, respectively. Twenty to forty cells per sensory cluster were injected with DNA. The injected ganglion was incubated with L15 medium containing 50 μM 5-HT, 500 μM IBMX (0.1% dimethyl sulfoxide) for 17 hr, and noninjected ganglion from the same animal was incubated with L15 medium containing 0.1% dimethyl sulfoxide as control.

Ten minutes after DNA injection, a pair of sensory clusters were incubated with L15 medium (Schacher and Proshansky, 1983) containing drugs or vehicle control (0.1% dimethyl sulfoxide for IBMX) either continuously for 2 hr or in 5 min pulses, one to five times, with 15 min intervals. After incubation at 16°C, ganglia were rinsed with L15 medium and incubated 3 hr before X-gal (GIBCO BRL) staining or harvesting. Fixation and staining with X-gal were done as described by Kaang et al. (1992). To prepare ganglionic extracts, pleural ganglia were vortexed (5–10 min) in 60 μl or 30 μl of TT buffer (250 mM Tris [pH 7.5], 0.4% Triton X-100) for assay of either CAT or β-galactosidase, respectively (MacGregor et al., 1991).

Assay of Reporter Gene Expression

β-Galactosidase activity was measured by the fluorometric assay using MUG (4-methylumbelliferyl-β-D-galactoside) at 37°C for 11 hr or at room temperature for 30 min and 30 μl of cell extract from one ganglion as described by MacGregor et al. (1991). Fluorescence measurement for the MUG assay was done using Titertek Fluoroskan II (Flow Laboratories, Inc.) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm in a 96-well plate (catalog number 011-010-7801; Dynatech Lab, Inc.). The relative β-galactosidase activity of a single cell was calculated as follows: $\beta_s = (\beta_i - \beta_o)/N$ (β_s, single cell activity; β_i, total fluorescence of injected ganglion; β_o, fluorescence background from noninjected ganglion control; N, number of neurons injected). Single cell activities from the injected ganglion and noninjected ganglion of the same animal were compared and statistically analyzed using two-tailed paired t test.

The fluorescent CAT assay was done using FAST-CAT (#F-2900, Molecular Probes, Inc.). The reaction was performed at 37°C for 11 hr, using 60 μl of cell extract from one ganglion. Quantitation of CAT activity (percentage of conversion ratio) was done as described in the instruction manual by measuring fluorescence of substrate and products from each CAT reaction using Perkin-Elmer LS-5B luminescence Spectrometer at an excitation wavelength of 490 nm and an excitation slit of 15 nm, and at an emission wavelength of 512 nm and an emission slit of 5 nm. CAT

activity of single neurons was calculated by dividing total CAT activity of single pleural ganglion by the number of DNA-injected neurons.

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